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Synovial fibroblasts as target cells for staphylococcal enterotoxin-induced T-cell cytotoxicity

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SUMMARY

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown aetiology. Recently, superantigens have been implied in the pathogenesis of RA. Superantigens activate a large fraction of T cells leading to the production of cytokines and proliferation. In addition, superantigens direct cellular cytotoxicity towards major histocompatibility complex (MHC) class II-expressing cells. There is now increasing evidence that cytotoxic T cells may be involved in the pathogenesis of RA. In the inflamed synovia class II-positive synovial fibroblasts (SFC) are found. In the present study it was tested whether MHC class II-positive SFC serve as target cells for superantigen-induced cellular cytotoxicity. SFC were stimulated with interferon-γ to express class II antigens, then they were cultivated in the presence of CD4-positive T cells with or without staphylococcal enterotoxins (SE). Cytotoxicity of T cells was measured as release of lactate dehydrogenase from SFC. Specific cytotoxicity was only found in the presence of class II-positive SFC depending on the dose of SE. Maximum lysis was seen after 20 hr. T-cell cytotoxicity was inhibited by antibodies to MHC class II antigens. The data suggest that class II-positive SFC not only function as accessory cells for SE-mediated T-cell proliferation and interleukin-2 production but may also be the targets of superantigen-mediated cellular cytotoxicity.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown aetiology. T cells are believed to play an important role in the pathogenesis of RA. They infiltrate the joints and express several activation markers including human leucocyte antigen (HLA)-DR and interleukin-2 (IL-2) receptor.[1,2] In several studies it has been reported that T cells bearing specific Vβ chains are enriched in the inflamed synovia of RA patients, pointing to T-cell activation by superantigens.[3,4] Superantigens, such as staphylococcal enterotoxins (SE), are a group of related proteins that bind to major histocompatibility complex (MHC) class II molecules outside the antigen-binding groove[5] and to the Vβ region of the T-cell receptor (TCR).[6,7] By bridging MHC class II-bearing accessory cells with T cells expressing particular TCR Vβ sequences SE activate a large fraction of T cells leading to the production of cytokines [e.g. Il-2, interferon-γ (IFN-γ), tumour necrosis factor (TNF)] and proliferation.[8–10] In addition it is well known that SE can direct cellular cytotoxicity towards MHC class II-expressing cells. Such retargeting of T cells is referred to as staphylococcal enterotoxin-dependent cell-mediated cytotoxicity (SDCC) and results in killing of the cells presenting the SE–MHC class II complex.[11,12]

There is now increasing evidence that cytotoxic T cells may be involved in the pathogenesis of RA. In several studies synovial T cells of RA patients have been characterized using CD4 and CD8 cell surface markers. The CD4/CD8 ratio varies within the joint. In general, a predominance of CD4-positive T cells has been reported.[13,14] Expression of CD4, however, does not necessarily implicate 'helper function' of these cells. Both CD8-positive as well as CD4-positive T cells have been reported to exert cytolytic function.[15] Using *in situ* hybridization Griffiths *et al.* could identify in the synovial fluid of RA patients both CD4 and CD8-positive T cells expressing perforin and granzyme, functional markers of cytolytic cells.[16] Only recently, activation of cytotoxic cells in the synovial tissue of RA patients has also been reported.[17,18] In the inflamed synovia class II-positive synovial fibroblasts are found.[19] They may serve as possible target cells of CD4-positive cytotoxic cells.[20]

Recently we[21] and others[22,23] reported that MHC class II-positive synovial fibroblast cultures exhibit accessory functions for SE-mediated T-cell activation. Furthermore, Boots *et al.* could demonstrate that stimulated synovial fibroblast cultures also function as antigen-presenting cells for regular antigens.[24] Thus synovial fibroblasts may be active participants in the development of RA.

In the present study we have examined whether in addition

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to their role as SE-presenting cells synovial fibroblasts can also serve as target cells for SE-induced cellular cytotoxicity.

MATERIALS AND METHODS

Preparation and culture of synovial fibroblasts

Synovial fibroblasts were obtained from patients with degenerative joint disorder or RA. Adherent synovial cells were isolated according to the protocol of Dayer *et al.* with modifications.[25] The lining cell layers were separated from the synovial material, minced and digested with collagenase (850 U/ml, type Ia; Sigma, Deisenhofen, Germany) and hyaluronidase (3000 U/ml; Serva, Heidelberg, Germany) in Dulbecco's minimum essential medium (DMEM; Gibco, Berlin, Germany) containing 1% antibiotic/antimycotic (Gibco), for 2–4 hr. The cells were then sieved through sterile gauze, washed and seeded on 24-well plates (Greiner, Nürtingen, Germany) at a density of 10^5 cells/well. After 7–14 days in culture, when they had reached confluence, the cells were passaged onto new 24-well or 96-well plates. Synovial fibroblast cell cultures (SFC) usually were used for experiments between passages 3 and 7. At this time-point all cells had the appearance of fibroblasts. They were all positive for vimentin and stained negatively with anti-Leu-M3 and anti-HLA-DR.

Cytochemical characterization of SFC

SFC were characterized as described previously.[26] Briefly, cells were fixed to coverslips with ice-cold methanol. For characterization by indirect immunofluorescence, the following antibodies were used. Anti-HLA-DR (Dianova, Hamburg, Germany), anti-Leu-M3 (Becton-Dickinson, Heidelberg, Germany), anti-vimentin (Dakopatts, Hamburg, Germany) and anti-intracellular adhesion molecule-1 (ICAM-1) (Dianova). Expression of MHC-class II or B7 by synovial fibroblast cells after treatment with interferon-γ (IFN-γ 200 U/ml) was determined by staining cells with an anti-HLA-class II monoclonal antibody (mAb) or anti-B7 (CD80, CD86) (all obtained from Dianova) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Dianova). The samples were then analysed on a fluorescence activated cell sorter (FACScan)-cytofluorometer (Becton-Dickinson).

T-cell clones

The CD4+CD8$^-$, (TCR) α^+ β^+ T-cell clones D894 and A37/9 were established and propagated as described elsewhere.[27] Briefly, purified T cells from human peripheral blood were cultured at 0·3 cells per well in 96-well round-bottom culture plates (Nunc, Wiesbaden, Germany) in the presence of 10^5 irradiated (3000 rads) peripheral blood mononuclear cells (PBMC) and 1 μg/ml phytohaemagglutinin P (PHA; Wellcome, Burgwedel, Germany). Growing clones were expanded in RPMI-1640 (Biochrom KG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, antibiotics, 10% heat-inactivated fetal calf serum (complete medium) and 30 U/ml recombinant IL-2 (EuroCetus, Amsterdam, the Netherlands). The clones were restimulated every 2–3 weeks with a feeder cell mixture of irradiated PBMC and irradiated Epstein–Barr virus-transformed lymphoblastoid cell lines and PHA-P.[27]

Experimental procedures

SFC grown in 96-well plates were cultured in the absence or presence of IFN-γ (200 U/ml) for 5 days, before they were washed with RPMI complete medium. T cells (clone D894 or A37/9) were added at 2×10^4–10×10^4 cells per well in the absence or presence of staphylococcal enterotoxin E (SEE) (Serva, Heidelberg, Germany) at concentrations of 0·1–30 ng/ml in a final volume of 200 μl. After 24 hr, supernatants were harvested, centrifuged and tested for lactate dehydrogenase (LDH) activity. Monoclonal antibodies anti-HLA class II and anti-HLA class I (Dianova) were preincubated with SFC for 15 min before T cells were added.

Cytotoxicity assay

Cytotoxicity was determined by measuring the LDH activity in the supernatants with a photometrical assay (LDH optimized EC 1.1.1.27 UV.Test; Sigma, Deisenhofen, Germany). Supernatants of each sample were harvested centrifuged and LDH-activity in the supernatants was determined with a photometer (wavelength 340 nm) (Spectrometer Ultrospec III, Pharmacia, Freiburg, Germany). The oxidation of reduced nicotinamide adenine dinucleotide (NADH) to NAD^+ as equimolar amount of reduction of pyruvate to L-lactate was continuously recorded over 5 min with a graph-recorder (REC 101, Pharmacia, UK) and calculated as increase of *x*-fold over 5 min. Spontaneous release was determined by incubation of target cells in medium alone, maximum release by incubation with 1% Triton-X-100 (Sigma). The per cent specific lysis was calculated by using the following formula:

$$100 \times \frac{LDH_{exp.} - LDH_{spont}}{LDH_{max.} - LDH_{spont}}$$

Determination of IL-2 production

Ten to 14 days after addition of feeder cells and PHA, T cells were washed and cultured at $5 \times 10^+$–10×10^4 cells per well with SFC in 96-well plates in the absence or presence of SEE. After 24 hr, cell-free supernatants were tested for IL-2 content using IL-2-dependent murine cytoxic T-lymphocyte line (CTLL) cells. Serial dilutions of the test supernatants were added to 3×10^4 CTLL cells for 24 hr. The viability of the CTLL cells was measured in a colorimetric assay (cleavage of tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide [MTT]) as described.[28]

RESULTS

Staphylococcal enterotoxins (SE) direct T cells to mediate cytotoxicity against HLA class II-positive SFC

Recently we could demonstrate that class II-positive SFC function as accessory cells for SE-mediated T-cell activation.[21] SFC cultured *in vitro* for several passages did not express class II antigens nor B7. To induce class II expression SFC were cultivated with IFN-γ. After 3–5 days more than 90% of SFC acquired MHC class II, but not B7. Class II-positive cells were cultured with SE-reactive CD4+CD8−, $\alpha\beta^+$ T-cell clones and SE. In the presence of class II-positive SFC we could demonstrate SE-mediated T-cell proliferation after 48 hr and IL-2 production after 24 hr. After 24 hr we also noticed that the characteristic SFC monolayer has disappeared

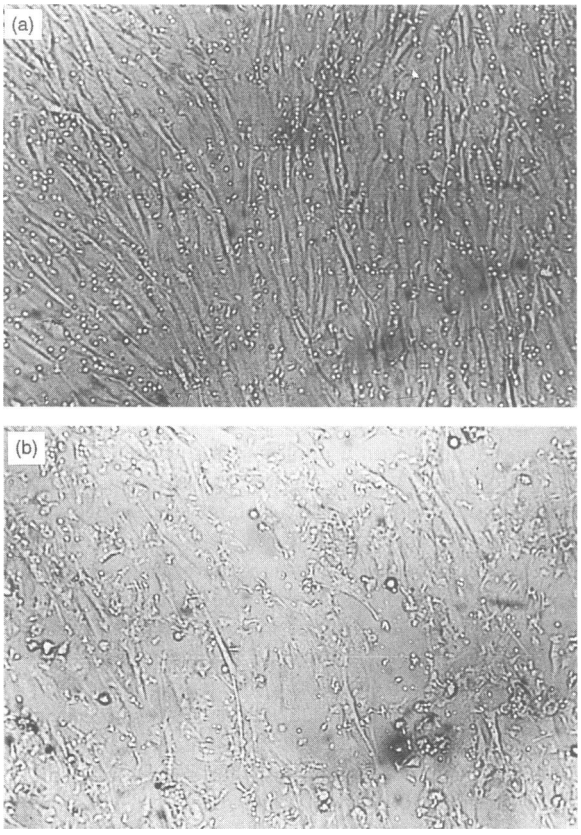

Figure 1. Class II-negative SFC (a) or class II-positive SFC (b) were cultured with 5×10^4 T cells (clone A37/9) in the presence of SEE 10 ng/ml. After 24 hr the cell monolayer of class II-positive SFC was destroyed (b) whereas class II-negative SFC still showed their characteristic morphology.

(Fig. 1a, b) when class II-positive SFC were cultured with T cells in the presence of SE (Fig. 1b). The SFC monolayer was not altered in the absence of SE or when class II-negative SFC were used (Fig. 1a) suggesting that HLA class II-positive SFC not only served as accessory cells for SE-induced T-cell activation but also as target cells for SE-induced T-cell cytotoxicity.

To test this hypothesis further HLA class II-positive SFC were cultured with a SEE-reactive T-cell clone in the absence or presence of SEE (10 ng/ml). Cytotoxicity was determined by measuring LDH activity in the cell supernatants. Specific cytotoxicity was only seen when class II-positive SFC were cultured with T cells in the presence of SEE (Table 1) whereas in the absence of SEE no significant increase in cytotoxicity could be measured. No increase in cytotoxicity was observed in the absence of T cells indicating that the cytotoxicity measured was T cell-dependent. In addition no, or only a weak, cytotoxic reaction could be measured in the presence of class II-negative SFC suggesting that class II-positive SFC were necessary as accessory cells for SE-induced T cell-mediated cytotoxicity. Throughout all experiments a minimal 2·4-fold to a maximal 25-fold increase in specific cytotoxicity was measured when comparing class II-positive versus class II-negative cells. Furthermore, no significant LDH activity was measurable when T cells were stimulated with SEE in the absence of SFC, indicating that autokilling of T cells does not appear to play a role in our experimental set up.

Table 1. SEE directs T cells to mediate cytotoxicity against HLA class II-positive SFC

	Specific lysis (%)
Class II-positive SFC + T cells + SEE	31·7
Class II-positive SFC + T cells + SEE*	46·5
Class II-positive SFC + T cells	6·6
Class II-positive SFC	4·8
Class II-negative SFC + T cells + SEE	11·5
Class II-negative SFC + T cells + SEE*	12·4
Class II-negative SFC + T cells	11·0
Class II-negative SFC	6·5
T cells + SEE 10 ng/ml	5·7
T cells	4·4

SFC cultured with or without IFN-γ for 5 days were incubated with 5×10^4 T cells/ml (clone A 37/9) in the presence or absence of SEE 10 ng/ml. Cytotoxicity was measured as increase in LDH activity in the cell supernatants. One of five independent experiments is shown.
*Preincubation of SFC with SEE for 20 hr.

SE-dependent cellular cytotoxicity (SDCC) was slightly increased when class II-positive SFC were preincubated with SEE for 20 hr. Table 1 shows that after preincubation with SEE a 1·6-fold increase in specific lysis was measured. Preincubation of SFC with SEE had no effect on SE-mediated T cell-dependent cytotoxicity when SFC were not IFN-γ treated.

T-cell-mediated lysis of MHC class II-positive SFC is time and dose dependent

To test further SDCC against class II-positive SFC, IFN-γ treated SFC preincubated with SEE were cultured with T cells and SEE in different concentrations and for various time periods. In Table 2 it is shown that specific LDH release was measurable after 12 hr. After 20 hr maximal LDH release was seen and no further increase was measured at later time-points. Again, no or only minor cytotoxicity was observed when T cells and SEE were incubated with SFC not stimulated with IFN-γ, suggesting that class II expression on SFC was a prerequisite for SE-mediated T cell-dependent cytotoxicity. No significant LDH release was measured when class II-positive SFC were cultured with SEE for 20 hr in the absence of T cells. Furthermore, no significant LDH release into the supernatant occurred when T cells were cultured alone or with SEE. These results indicate that the LDH measured in the supernatants derived from SFC. This was further supported by the observation that even treatment of T cells with 1% Triton-X-100 did not exceed the spontaneous release from SFC.

The SE-dependent cellular cytotoxicity was not only time dependent but also dose dependent. This is shown in Table 2 and Table 3. With increasing amounts of SEE an increase in cytotoxicity could be measured.

Influence of different effector to target ratios on SDCC

So far it could be shown that class II-positive SFC not only function as accessory cells for SE-mediated T-cell activation

Table 2. Staphylococcal enteroxtoxin-dependent T cell-mediated cytotoxicity against SFC is time and dose dependent

Incubation with:	% specific cytotoxicity SFC with IFN-γ	SFC without IFN-γ
T cells + SEE 1 ng/ml		
5 hr incubation	2·13	ND*
12 hr incubation	10·65	3·55
20 hr incubation	19·2	5·06
T cells + SEE 10 ng/ml		
5 hr incubation	2·13	ND
12 hr incubation	16·96	5·68
20 hr incubation	46·5	12·43
T cells (20 hr incubation)	6·57	11·0
SEE 10 ng/ml (20 hr incubation)	4·79	ND

SFC cultured with or without IFNγ 200 U/ml were cultured with 5×10^4 T cells/well of the CD4+CD8− clone A37/9 in the presence of SEE 1 ng/ml or 10 ng/ml for various time periods. Cytotoxicity was measured as release of LDH activity into the cell supernatants. (Specific lysis; T cells alone, 4·44%; T cells + SEE 1 ng/ml, 5·06%; T cells + SEE 10 ng/ml, 5·6%). One of three independent experiment is shown.

*ND, not detectable.

Table 3. SEE-dependent T cell-mediated cytotoxicity against SFC correlates with IL-2 production from T cells

	% specific cytotoxicity	IL-2 production (U/ml)
SEE 0·1 ng/ml	ND*	ND*
SEE 3 ng/ml	36·4	2·6
SEE 30 ng/ml	60·4	9·1

Class II-positive SFC were cultured with T cells (clone A37/9) in the presence of increasing concentrations of SEE. After 24 hr, cell supernatants were harvested and centrifuged before they were tested for LDH activity and IL-2.

*ND, no measurable specific cytotoxicity or IL-2 detectable, respectively.

but can also be the targets of T-cell cytotoxicity. Next, we tested how the effector to target ratio can affect SDCC. IFN-γ-treated SFC were cultured with different concentrations of T cells in the presence of 10 ng/ml SEE. In Table 4 it is shown that after 10 hr of incubation a measurable release of LDH was only seen at an effector to target ratio of 10:1. After 24 hr however a low but significant LDH release was seen at an effector to target ratio of 1:1. Again, SE-mediated cytotoxicity only occurred in the presence of IFN-γ treated SFC. With class II-negative SFC no, or only a small, release of LDH was seen even at high effector to target ratios.

Inhibition of SE-induced T-cell-mediated cytotoxicity by anti-HLA class II mAb

SE-induced T cell-mediated cytotoxicity correlated with the expression of class II molecules on SFC (see Table 1 and 2). SE-induced T cell-mediated cytotoxicity against SFC occurred

Table 4. Influence of different effector to target ratios on SDCC

	1×10^4 T cells	3×10^4 T cells	1×10^5 T cells
% specific cytotoxicity after 10 hr			
SFC with IFN-γ	ND*	2·4	16·1
SFC without IFN-γ	ND	ND	10·8
% specific cytotoxicity after 24 hr			
SFC with IFN-γ	17·8	24·9	46·9
SFC without IFN-γ	ND	ND	19·3

SFC (10^4/well) stimulated with or without IFNγ were cultured with various concentrations of T cells (clone D894) in the presence of SEE 10 ng/ml. Specific cytotoxicity was measured after 10 hr or 24 hr respectively as release of LDH into the cell supernatant.

*ND, no specific cytotoxicity detectable.

only in the presence of IFN-γ stimulated SFC. These results suggested that class II expression was a prerequisite for SDCC against SFC. To test this hypothesis IFN-γ treated SFC or SFC not treated with IFN-γ were preincubated with anti-class II mAb for 15 min. Then T cells were added with or without SEE. Figure 2 shows that increasing concentrations of mAb against MHC class II blocked specific cytotoxicity up to more than 90%. Monoclonal antibodies against MHC class I had no effect on the specific LDH release indicating that interaction between T cells and MHC class I did not play a major role in our experimental system. Again, no cytotoxicity was measured when T cells or SFC were cultured alone or in the presence of SEE.

Correlation of SDCC and IL-2 release

To determine whether SDCC was paralleled by IL-2 production we compared specific lysis with IL-2 concentrations in the supernatants of the test samples. IFN-γ treated and untreated SFC were cultivated with T cells in the presence of increasing amounts of SEE. As shown in Table 3 specific cytotoxicity increased with increasing concentrations of SEE from 0% (SEE 0·1 ng/ml) to 60·4% (SEE 30 ng/ml). With unstimulated SFC no specific cytotoxicity was detectable. Parallel to the increase in cytotoxicity a dose-dependent increase in IL-2 production was seen from undetectable amounts at 0·1 ng/ml up to 9·07 U/ml with 30 ng/ml. The data suggest that killing of SFC was accompanied by activation of the T cells.

DISCUSSION

Recently we showed that class II-positive SFC function as accessory cells for SE-dependent T-cell proliferation and IL-2 production.[21] In addition to the binding of the MHC class II enterotoxin complex to the TCR, interaction of ICAM-1 to lymphocyte function-associated antigen-3 (LFA-3) as a second signal was required for a successful T-cell activation. Interaction of B7 with CD28, which appears to be an important signal,[29] is improbable as a second signal, since B7 was not detectable on SFC.

In the present paper we demonstrate that class II-positive SFC are also the targets of SE-dependent cellular cytotoxicity mediated by CD4-positive T cells. The observation that CD4-positive T cells exert cytotoxic function is not surprising.

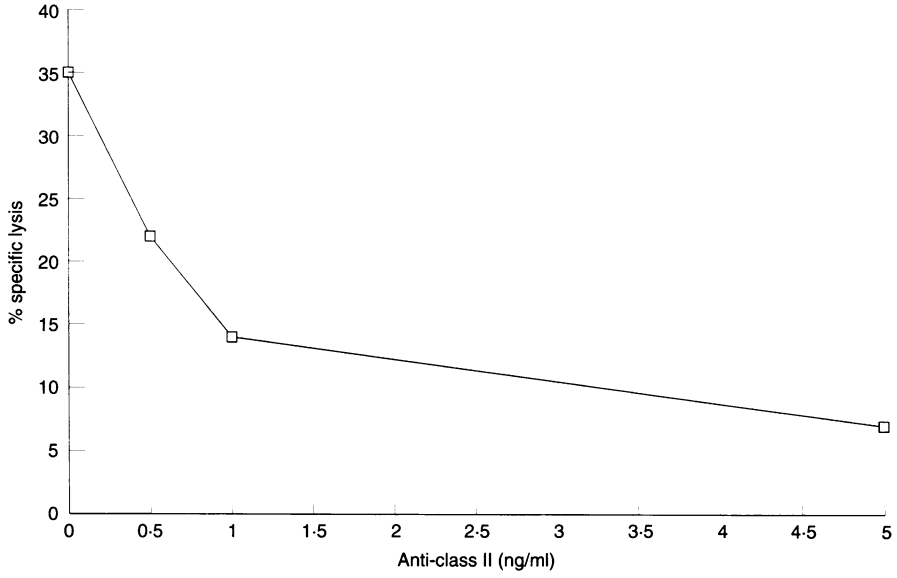

Figure 2. Inhibition of T cell-mediated cytotoxicity against IFN-γ-stimulated SFC by mAb against MHC class II. Class II-positive SFC were preincubated with various concentrations of mAb against MHC class II for 15 min before 5×10^4 T cells (clone D894) and SEE 1 ng/ml were added to each well. Specific cytotoxicity was measured after 24 hr. With a mAb against MHC class I (5 ng/ml) no decrease in specific cytotoxicity was measured (34.7%). One of three experiments is shown.

In several studies it has been shown that CD8-positive as well as CD4-positive T cells may exert cytotoxic activity against target cells.[15,30,31] There are also reports that CD4-positive T cells predominate in the synovial tissue of RA patients and that these T cells have a cytotoxic potential.[32]

SDCC is a well-studied phenomenon.[11] In most studies cancer cells, B cells, leucocytes or monocytes are used as target cells.[12,33] There is only little known about tissue cells as targets for cytotoxic T cells. So far, lysis of chondrocytes and keratinocytes by lymphokine-activated killer cells has been reported.[34,35] We now extend these observations by studying class II-positive SFC as targets of SDCC.

SE-mediated cytotoxicity against SFC was strongly dependent on class II expression. Only with IFN-γ stimulated SFC a cytotoxic reaction could be measured and this was inhibited by a mAb directed against HLA class II. This implicates cytotoxicity against SFC based on a MHC class II-restricted direct cell interaction as it has been shown for SDCC in other systems. Although SE-dependent T-cell autokilling has been described by others[36] we did not observe this phenomenon in our experimental system. There have been also reports about MHC-independent SDCC.[37] Under our experimental conditions, however, T cell-mediated cytotoxicity was only measured in the presence of class II-positive SFC.

The underlying mechanism of SDCC against SFC was not the aim of this study and needs further investigation. One possible mechanism of lysis by cytotoxic T cells could be the release of perforin or granzymes. Perforin and granzyme A have been found in T cells of the synovia of RA patients pointing to a possible role of cytotoxic T cells for the pathogenesis of RA.[16] A further mechanism for cell lysis described in the literature is loss of adhesion.[38] This mechanism is based on a T cell-mediated detachment of adherent growing cells. This detachment usually precedes the destruction of the cells. We observed detachment of SFC already after 5 hr whereas maximal cytotoxicity occurred only after 20 hr suggesting that loss of adhesion is not the only mechanism involved in SDCC against SFC. Loss of attachment, however, might render cells more sensitive towards cellular cytotoxicity.

Only recently it was shown that the surface molecules fibroblast-associated surface antigen (FAS) and its ligand FAS-L are involved in the function of cytotoxic T cells. It could be shown that FAS is involved in T cell-mediated cytotoxicity of MHC class II-transfected dermal fibroblasts.[31] Thus FAS may also play a role in SDCC of SFC.

There are several reports showing that cytotoxic T cells play a role in RA. Person *et al.* demonstrated that the cytotoxic activity of peripheral T cells from RA patients is increased compared to that in healthy individuals. In addition, peripheral leucocytes of RA patients have an increased cytotoxic activity against synovial fibroblasts of RA patients compared to synovial fibroblasts of healthy individuals.[20] By *in situ* hybridization Griffith *et al.* demonstrated cytolytic lymphocytes in the SF of patients expressing granzyme A and perforin.[16] Miltenburg *et al.* studied T-cell clones derived from the synovia of a patient with RA. They demonstrated that these clones have the functional characterization of a Th1 type.[32] Th1 cells differ from Th2 cells in their cytotoxic potential and their capacity to produce IL-2 and IFN-γ, whereas Th2 T-cell clones produce IL-4 and IL-5.[39] Th1 cells may be activated when encountering their specific antigen however they could also be activated via superantigens. The data demonstrate that putative cytolytic lymphocytes capable of mediating target cell destruction are present at the site of inflammation. Our studies suggest that SFC are possible target cells for cytotoxic T cells. Destruction of SFC may lead to a release of so far hidden antigenic peptides which then might be recognized by T cells. In addition, lysed SFC might be phagocytosed by macrophages which in turn release inflammatory mediators leading to a further increase of inflammatory cells into the synovial tissue. Thus cytotoxicity of T cells against SFC may initiate or perpetuate the inflammatory process in RA.

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